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Lipid-Protein Interactions via Fluorescence Quenching in Small Unilamellar Vesicles

Talia Kordahi
The University of Akron, tnk13@zips.uakron.edu

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Abstract

Studying peptide interactions with model membranes have aided in understanding protein-lipid interactions more clearly. In this report, fluorescent small unilamellar vesicles (SUVs) of a known composition were made. Two peptides, RND1 and MARCKS, were also fluorescently labeled and added to a solution of SUVs. The interactions between these were observed via fluorescence quenching, read out with a spectrofluorimeter. In addition, two environments of buffer were tested: 1X PBS and 0.1X PBS. After obtaining data, it was concluded that MARCKS quenched the SUVs more than RND1. This could be due to the presence of more lysine amino acids in MARCKS, which have been known to play a role in membrane protein activity (Li et al., 2013). No significant changes were observed when the buffer conditions were manipulated.

Introduction

Each cell of our body is surrounded by a lipid bilayer composed of amphiphilic phospholipids, regulating the intracellular molecules and diffusion (Alberts et al., 2002). Studying the lipid bilayer can give insight into the lipid-protein interactions, eventually expanding the fields of cell signaling and drug delivery pathways. This research project will focus on small unilamellar vesicles (SUVs) of a known composition and their interaction with two different peptides, RND1 and MARCKS, in two varying buffered environments.

The SUV composition being observed will be 96.4% 1,2-dioleoyl-sn-glyerco-3-phosphocholine (DOPC), 3.5% 1,2-dioleoyl-sn-glyerco-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (DOGS-NTA), and 0.1% 1-palmitoyl-2-(dipyrromethene boron difluoride)undecanoyl-sn-glyerco-3-phospho-L-serine (TopFluor PS). DOPC is chosen because phosphocholine is a main lipid component in animal cell membranes, it plays a role in producing phosphatidic acid and lysophosphatidic acid signaling lipids, and it is readily available in Dr. Smith's lab (Alberts et al., 2002). DOGS-NTA is chosen as a chelating head group to bind the histidine-tagged RND1 and MARCKS peptides. Lastly, TopFluor PS is chosen as the fluorescent lipid to fluorescently tag the SUVs for observation. The structures for each component are seen below.

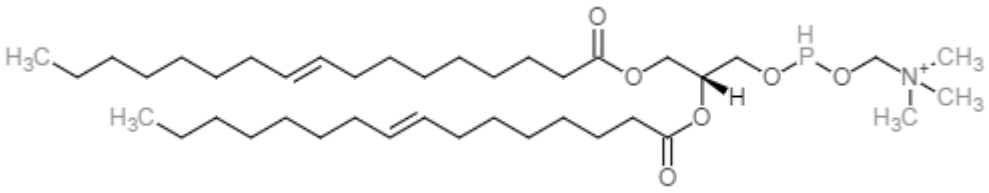

Figure 1: Structure of DOPC.

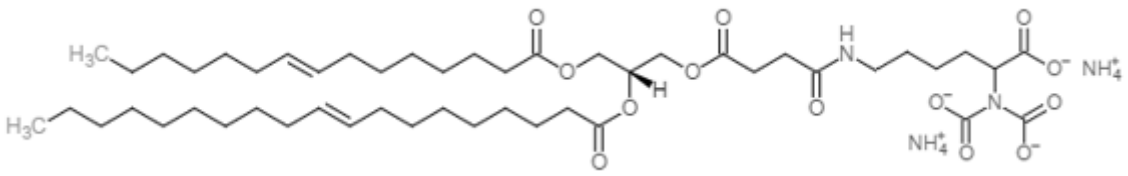

Figure 2: Structure of DOGS-NTA.

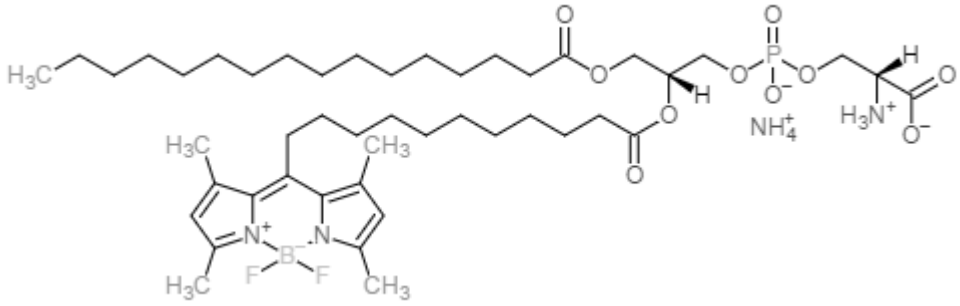

Figure 3: Structure of TopFluor PS.

The first peptide that will be used in this experiment is from the protein RND1, a 25 kDa protein of the Rho family GTPases (Ridley, 2006). This family of GTPases contains 20 members and affects cytoskeletal actin dynamics and cellular morphology by regulating cell proliferation, apoptosis, and gene expression (Zhu et al., 2013). It is mainly expressed in the brain and one major function is to control rearrangements of the actin cytoskeleton (Nobes et al., 1998).Other activities include regulation in epithelial adhesion, cell migration, membrane traffic, and tumor suppression (Okada et al., 2014). In this case, an N-terminal peptide fragment of RND1 that contains a Histidine tag on the N-terminus will be used; this specific peptide amino acid sequence is HHHHHHPQKSPVRSLSKRLL (GeneCard, 2016). In order to see the effect it has when incorporated into an SUV solution, RND1 is fluorescently dyed with the fluorophore 5-carboxytetramethylrhodamine (TAMRA).

The second peptide that will be used is from the protein myristoylated alanine-rich C-kinase substrate (MARCKS), a 32 kDa protein encoded by the MARCKS gene (Aderem, 1996). This

protein is known to be myristoylated, where it has a myristic acid derivative covalently attached to the α-amino group of the N-terminal of a glycine residue (Aderem, 1996). Like RND1, this protein may be associated with cell motility, membrane trafficking, and actin dynamics while unphosphorylated (Aderem, 1996). In this case, a His-tagged peptide fragment will also be used; this specific amino acid sequence is HHHHHHKKKKKRFSFKKSFKLSGFSFKKNKK. MARCKS will also be fluorescently dyed with TAMRA to quantitatively analyze the interactions, if any, with the SUVs.

The purpose of the experiment is to see if there will be interactions between SUVs and the chosen peptides RND1 and MARCKS. The fluorescence quenching of intensity can give insight on how many molecules are fluorescing as well as how much binding and interactions are occurring. It has also been seen that peptide binding to lipids is stronger at lower concentrations of salt (Kandasamy and Larson, 2006 and Shi et al., 2015). This is will also be tested by rehydrating the SUVs in two different concentrations of PBS: 1X and 0.1X. It is hypothesized that there will be more interaction between the SUVs and MARCKS in the 0.1X PBS environment due to the heavily lysinated amino acid sequence of MARCKS.

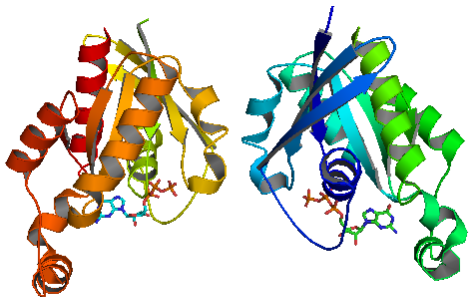

Figure 4: Ribbon diagram of the crystal structure of the human RND1 GTPase in the active GTP bound state (RCSB Protein Data Bank: 2CLS).

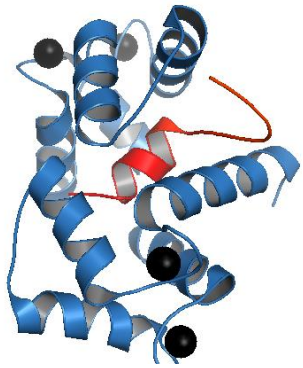

Figure 5: Ribbon diagram of the crystal structure of MARCKS calmodulin binding domain peptide complexed with calcium/calmodulin (RCSB Protein Data Bank: 1IWQ). The blue represents the calmodulin protein, the black represents calcium molecules, and the red represents MARCKS peptide.

Materials and Methods

Production of SUVs. The following components were ordered and obtained to make small unilamellar vesicles: 1,2-dioleoyl-sn-glyerco-3-phosphocholine (DOPC) (Avanti Polar Lipids, Inc. #850375C), 1,2-dioleoyl-sn-glyerco-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (DOGS-NTA) (Avanti Polar Lipids, Inc. #790528C), and 1-palmitoyl-2-(dipyrrometheneboron difluoride)undecanoyl-sn-glyerco-3-phospho-L-serine (TopFluor PS) (Avanti Polar Lipids, Inc. #810283C) with a composition of 96.4% DOPC, 0.1% TopFluor PS, and 3.5% DOGS-NTA. A 25 mL round bottom flask was obtained, cleaned with water and soap, and rinsed thoroughly with isopropyl alcohol. The flask was taken to the hood and piranha etched using sulfuric acid and 30% hydrogen peroxide in a 3:1 ratio, respectively. The flask was left to sit for 15 minutes. After 15 minutes, the flask was emptied, washed thoroughly with water, and put in a drying oven until no more water was present. The DOPC, DOGS-NTA, and TopFluor PS were obtained from the fridge, which were stored at -20˚C. The clean flask was rinsed three times with chloroform using a glass pipette, leaving a little chloroform on the third rinse. A 2 mL of 1 mg/mL solution of SUVs were made according to the desired lipid composition, as seen in **Table 1**. The desired volumes were dispensed into the chloroform in the

flask. The flask was taken to a rotary evaporator, put into a 44˚C water bath with a rotation of 128 rpm, and the chloroform was evaporated by decreasing the pressure. The flask was removed and dried using nitrogen gas. The SUVs were rehydrated with either 2 mL of 1X PBS or 2 mL of 0.1X PBS and were sonicated for 30 minutes to obtain a uniform diameter of ~60 nm. After sonication, the SUVs were transferred to a centrifuge tube and centrifuged for 10 minutes at 13.0 rpm.

Preparation of RND1, MARCKS, and PBS. A stock solution of RND1 was obtained and diluted to ~1 µM solution by pipetting 32.36 µL into 10 mL of PBS. The MARCKS stock solution was used as obtained. A 1X PBS solution was made from a 10X stock solution by adding 1 mL of the 10X PBS solution to 9 mL of water. A 0.1X PBS solution was made from a 10X stock solution by adding 0.1 mL of the 10X PBS solution to 9.5 mL of water.

SUV and Peptide Interactions. The concentrations of the SUVs and peptides were determined via Nanodrop. The determined concentrations for each trial are seen in **Tables 3 and 4**. A 10:1 ratio solution was made of peptide to SUV. Once the volumes of each solution were calculated, appropriate amounts of the peptide were added to the SUV solution. Fluorescence intensity was measured over a course of 30 minutes. This was repeated three times for each peptide and each concentration of PBS. The excitation wavelength chosen was 480 nm with an emission scan of 485-700 nm by increments of 1 nm and a band width of 5 nm. Three trials were ran for each peptide and each buffer environment and averaged. The averages are seen in **Tables 5-8.**

Calculations

[SUV] from Absorbance (**Figure 6**)

Beer-Lambert Law: $A = \varepsilon Cl$

$C = (0.851370)/ (95{,}000\ M^{-1}\ cm^{-1})\ (1\ cm)$

$C = 8.97 \times 10^{-6}\ M$

[RND1] from Absorbance (**Figure 7**)
Beer-Lambert Law: $A = \varepsilon Cl$
$C = (0.034416)/ (89{,}000\ M^{-1}\ cm^{-1})\ (1\ cm)$
$C = 3.87 \times 10^{-7}$ M

[SUV] from Absorbance (**Figure 8**)
Beer-Lambert Law: $A = \varepsilon Cl$
$C = (0.760841)/ (95{,}000\ M^{-1}\ cm^{-1})\ (1\ cm)$
$C = 8.01 \times 10^{-6}$ M

[MARCKS] from Absorbance (**Figure 9**)
Beer-Lambert Law: $A = \varepsilon Cl$
$C = (0.040046)/ (89{,}000\ M^{-1}\ cm^{-1})\ (1\ cm)$
$C = 4.50 \times 10^{-7}$ M

Results and Discussion

The mole percentage of DOPC, DOGS-NTA, and TopFluor PS were 96.4%, 3.5%, and 0.1% respectively. As seen in **Table 1**, the moles were converted to volumes to make the SUVs. The volumes needed for DOPC, DOGS-NTA, and TopFluor PS to make 2 mL of SUVs were 380.94 µL, 92 .98 µL, and 46.69 µL, respectively. Once the SUVs were made, it was important to know the excitation and emission wavelengths for each dye as well as the molar absorptivity. **Table 2** gives these values for both TopFluor PS and TAMRA. The excitation wavelength, emission wavelength, and molar absorptivity for TopFluor PS were 495 nm, 503 nm, and 95,000 $M^{-1}\ cm^{-1}$, respectively. The excitation wavelength, emission wavelength, and molar absorptivity for TAMRA were 553 nm, 576 nm, and 89,000 $M^{-1}\ cm^{-1}$, respectively. Based on these values, the excitation wavelength chosen was 480 nm with an emission scan of 485-700 nm.

In order to determine the correct volumes of each peptide and SUVs to use, a NanoDrop was used. The absorbance of the peak was used with the Beer Lambert Law to determine concentration. As seen in **Figure 6**, the calculated concentration for the SUVs used in the RND1 experiments was 8.97×10^{-6} M with the peak absorbance at 499 nm. **Figure 7** gave a spectrum for RND1; the calculated concentration was 3.87×10^{-7} M with the peak absorbance at 554 nm.

The SUVs and MARCKS peptide were then looked at. As seen in **Figure 8**, the calculated concentration for the SUVs used in the MARCKS experiment was 8.01 x 10^{-6} M with the peak absorbance at 498 nm. As seen in **Figure 9**, the calculated concentration of MARCKS was 4.50 x 10^{-7} M with the peak absorbance at 557 nm. All calculations are seen in the Material and Methods section. **Tables 3 and 4** show the desired volumes of each SUV, RND1, MARCKS, and PBS buffer solutions.

Overall, minimal quenching of SUVs by both peptides was seen. As seen in **Figure 10**, the fluorescence intensity for the SUVs overall increased over the 30 minutes while the fluorescence intensity gradually decreased for the RND1 by a total of 19% over the 30 minute time period in the 1X PBS environment. It was concluded that the SUVs quenched the RND1; however, the RND1 fluorescence quenching observed could also be due to exposure to a light source or other human error. As seen in **Figure 11**, the fluorescence intensity of the SUVs decreased by a total of 3% while the RND1 fluorescence intensity decreased by a total of 5% in the 0.1X PBS environment. This could show that less salinity led to less interactions between the SUVs and RND1 peptide. The final percent difference for each RND1 experiment is seen in **Figure 14**.

In **Figure 12**, the SUVs' fluorescence intensity decreased by a total of 15% over the course of 30 minutes while the MARCKS fluorescence intensity decreased by a total of 31% at the end of the 30 minutes in the 1X PBS environment. Likewise, the SUV and MARCKS fluorescence intensities decreased by 14% and 34%, respectively, at the end of the 30 minute time period in the 0.1X PBS environment. This is seen in **Figure 13**. The salinity difference for the MARCKS experiments, however, showed no significant effect. The final percent difference for each MARCKS experiment is seen in **Figure 15**.

Table 1: Table of values needed to make the SUVs according to the composition of 96.4% DOPC, 3.5% DOGS-NTA, and 0.1% TopFluor PS.

Component	mol %	mol needed	M.W. (g/mol)	mg needed	[X] mg/mL	Volume needed (µL)
DOPC	96.4	2.42×10^{-3}	786.15	1.90	5	380.94
DOGS-NTA	3.5	8.80×10^{-5}	1057.02	9.30×10^{-2}	1	92.98
TopFluor PS	0.1	2.51×10^{-6}	928.93	2.33×10^{-3}	0.05	46.69

Table 2: Fluorescent dye properties of both TopFluor PS and TAMRA.

	TopFluor PS	TAMRA
Excitation Wavelength (nm)	495	553
Emission Wavelength (nm)	503	576
Molar Absorptivity (M^{-1} cm^{-1})	95,000	89,000

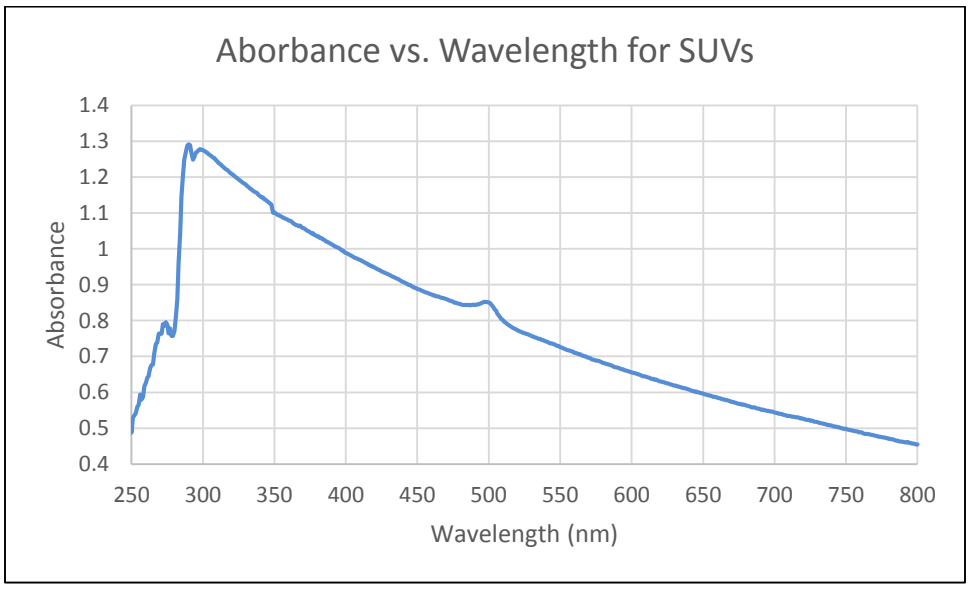

Figure 1: Absorbance vs. wavelength for the SUVs for the RND1 experiments. The calculated concentration was 8.97×10^{-6} M at 499 nm.

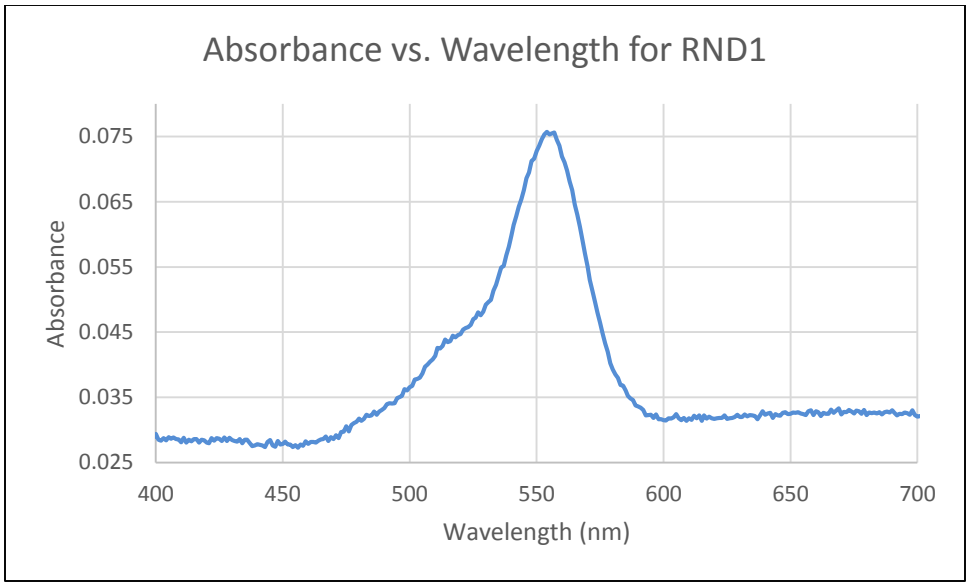

Figure 2: Absorbance vs. wavelength for RND1 peptide. The calculated concentration was 3.87 x 10^{-7} M at 554 nm.

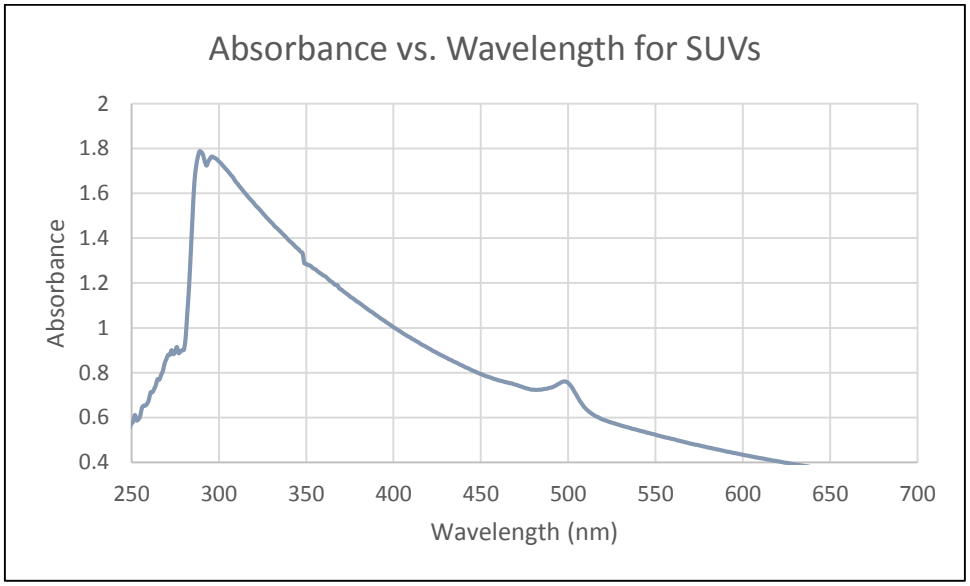

Figure 3: Absorbance vs. wavelength for the SUVs for the MARCKS experiments. The calculated concentration was 8.01 x 10^{-6} M at 498 nm.

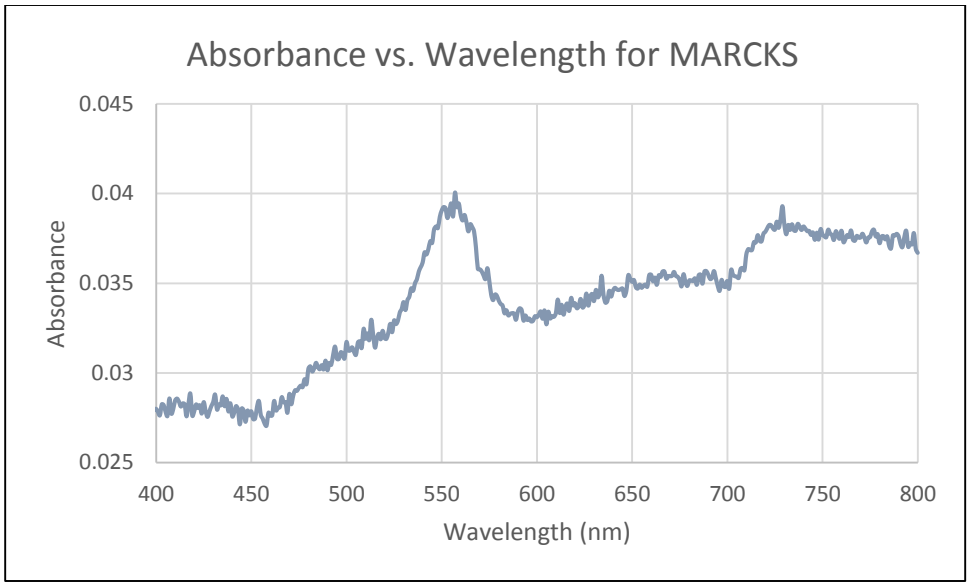

Figure 4: Absorbance vs. wavelength for the SUVs for the MARCKS peptide. The calculated concentration was 4.50 x 10^{-7} M at 557 nm.

Table 3: Table showing values of stock concentrations for SUVs and RND1, diluted desired concentrations, and volume needed of each for a total of 2 mL.

Stock (M)	
C_i (RND1)	4.50 x 10^{-7}
C_i (SUV)	8.01 x 10^{-6}
10:1 (M)	
C_f (RND1)	1.00 x 10^{-7}
C_f(SUV)	1.00 x 10^{-8}
Mixture (10:1) (L)	
V_i (stock SUV)	2.23 x 10^{-6}
V_i (stock RND1)	5.13 x 10^{-4}
PBS Buffer	1.48 x 10^{-3}

Table 4: Table showing values of stock concentrations for SUVs and MARCKS, diluted desired concentrations, and volume needed of each for a total of 2 mL.

Stock (M)	
C_i (MARCKS)	4.50×10^{-7}
C_i (SUV)	8.01×10^{-6}
10:1 (M)	
C_f (MARCKS)	1.00×10^{-7}
C_f(SUV)	1.00×10^{-8}
Mixture (10:1) (L)	
V_i (stock MARCKS)	2.50×10^{-6}
V_i (stock RND1)	4.45×10^{-4}
PBS Buffer	1.55×10^{-3}

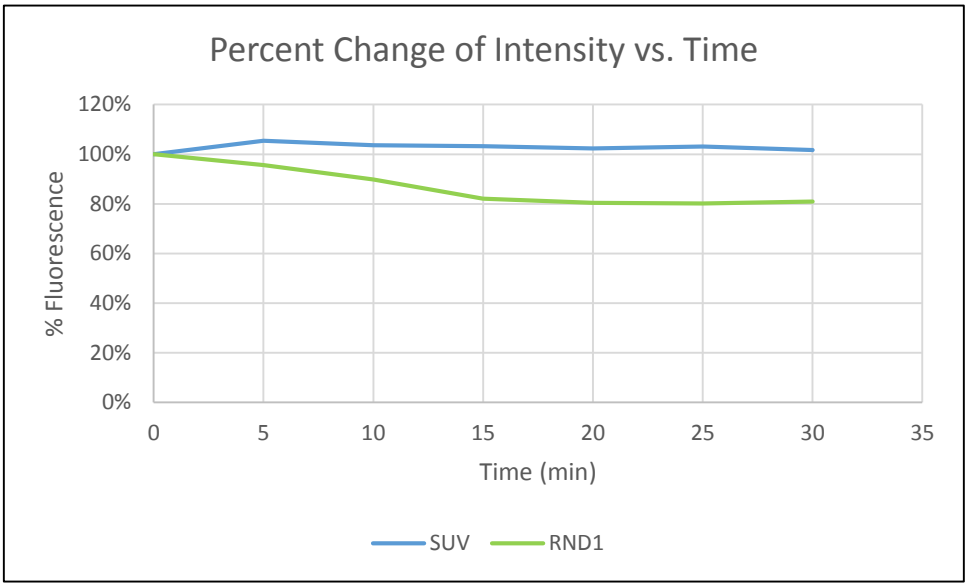

Figure 5: Graph representing the intensity changes of the SUVs and RND1 in a 1X PBS buffer over a time period of 30 minutes.

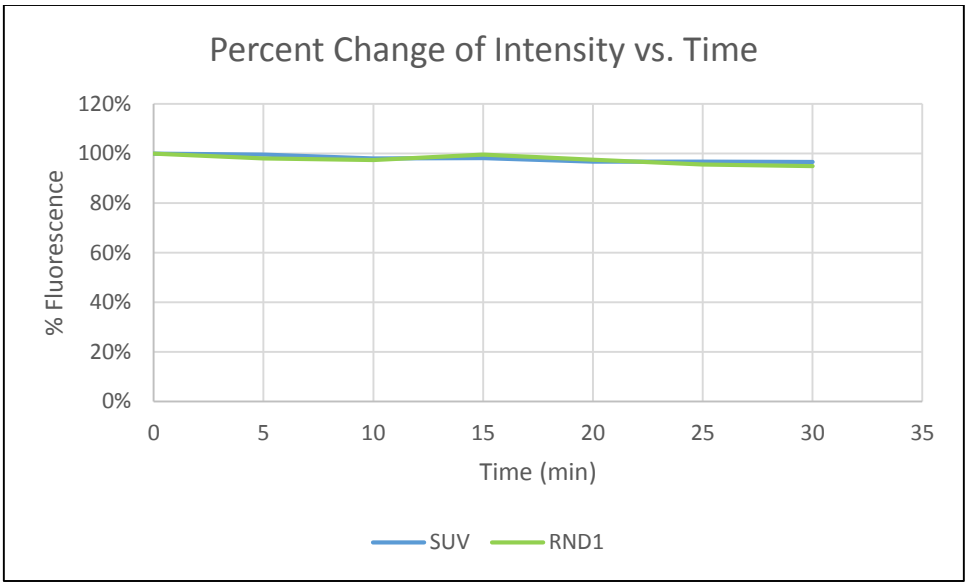

Figure 6: Graph representing the intensity changes of the SUVs and RND1 in a 0.1X PBS buffer over a time period of 30 minutes.

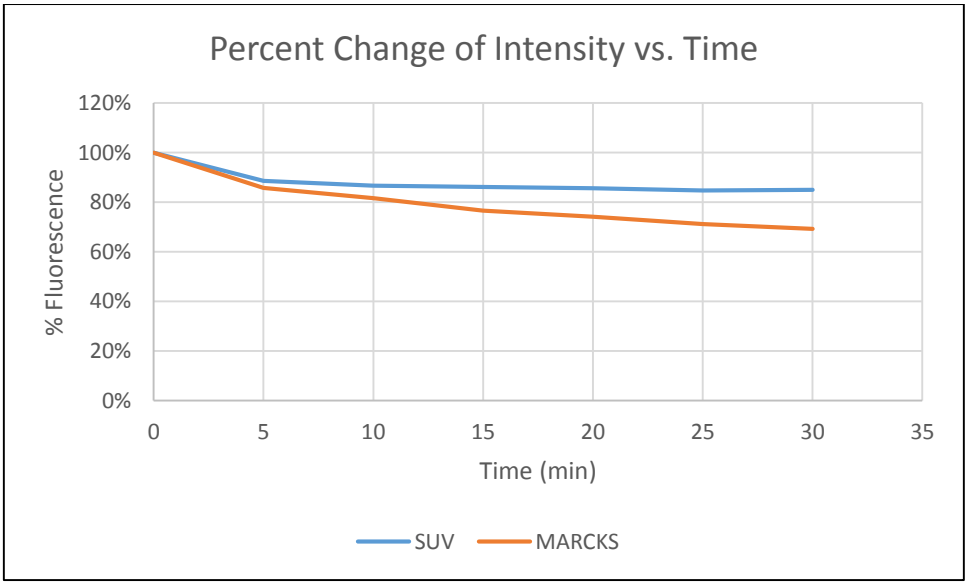

Figure 7: Graph representing the intensity changes of the SUVs and MARCKS in a 1X PBS buffer over a time period of 30 minutes.

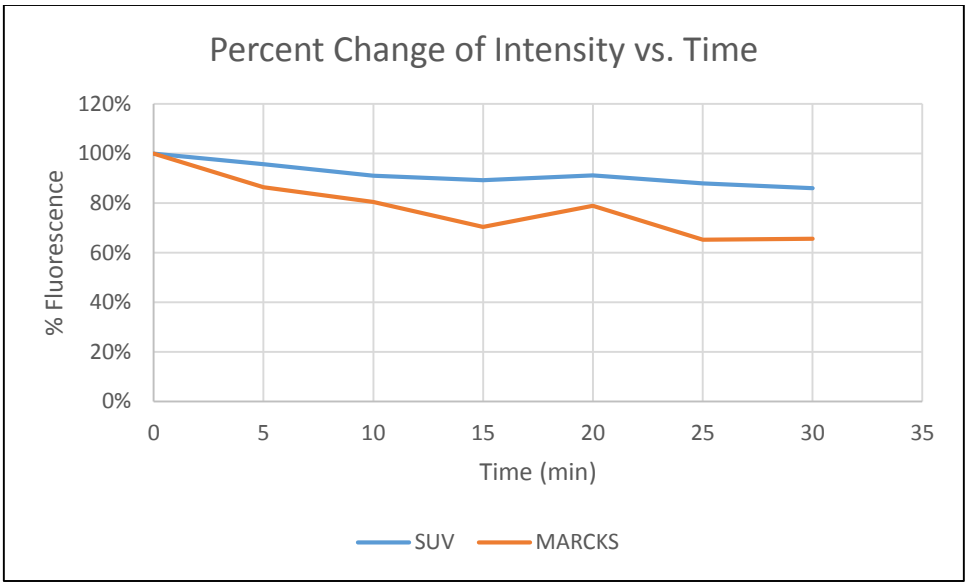

Figure 8: Graph representing the intensity changes of the SUVs and MARCKS in a 0.1X PBS buffer over a time period of 30 minutes.

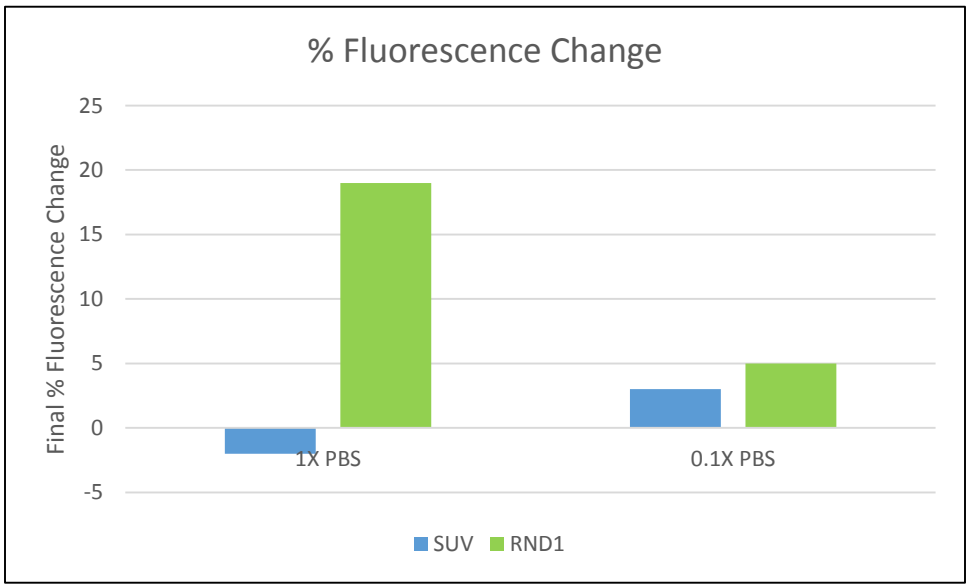

Figure 9: Bar plot showing final percent fluorescence change for the SUVs and RND1 in each PBS environment. The SUVs only quenched in the 0.1X PBS environment. RND1 quenched more in the 1X PBS environment.

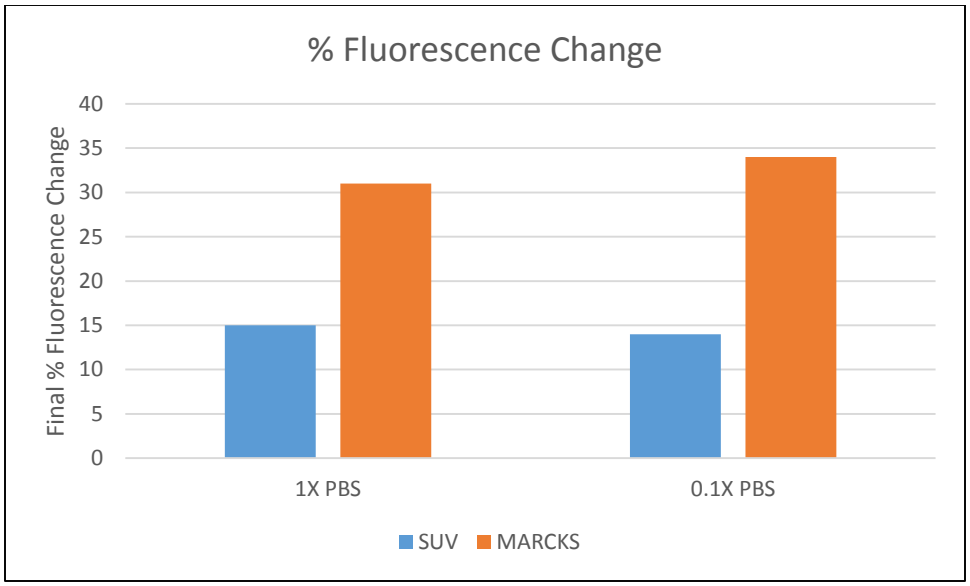

Figure 10: Bar plot showing final percent fluorescence change for the SUVs and MARCKS in each PBS environment. The SUVs quenched similarly, but more quenching was seen for the MARCKS in the 0.1X PBS environment

Conclusion

RND1 and its related proteins, RND2 and RND3, are all associated with actin cytoskeletal regulation; the N-terminal of each of these proteins is important in signaling (Oinuma et al., 2012). RND1 and RND3 contain an amino acid sequence, KERRA, which is unique to them (Oinuma et al., 2012). This amino acid sequence is involved in lipid raft-targeting (Oinuma et al., 2012). More SUV-RND1 interaction could have been seen if the RND1 peptide used still contained the KERRA sequence. Overall, the MARCKS peptide quenched the SUVs greater than the RND1 peptide. This could be due to the peptide sequence difference between RND1 (PQKSPVRSLSKRLL) and MARCKS (KKKKKRFSFKKSFKLSGFSFKKNKK). According to Li et al., lysine and arginine play crucial roles in membrane protein activity (Li et al., 2013). MARCKS contains 10 more lysine amino acids than RND1 although it contains one less arginine; these lysines could contribute to more interaction with the SUVs. Given more time and resources, different compositions of SUVs can be made and tested, offering more insight on protein-lipid interactions and their effect on cellular pathways inside the cell.

Works Cited

Aderem, A. The MARCKS family of protein kinase-C substrates. *Biochem. Soc. Trans.* [Online] **1996**, 23(3): 587-91.

Alberts, B; Johnson, A; Lewis, J; Raff, M; Roberts, K; Walter, P. Molecular Biology of the Cell, 4th Edition. Garland Science: New York, **2002**. Ch 10: The Lipid Bilayer.

Avanti Polar Lipids, Inc. 18:1 (Δ9-Cis) PC (DOPC). http://www.avantilipids.com/index.php?option=com_content&view=article&id=231&Itemid=492&catnumber=850375

Avanti Polar Lipids, Inc. 18:1 DOGS-NTA. https://www.avantilipids.com/index.php?option=com_content&view=article&id=856&Itemid=188&catnumber=790528

Avanti Polar Lipids, Inc. TopFluor PS. https://www.avantilipids.com/index.php?option=com_content&view=article&id=1964&Itemid=253&catnumber=810283

GeneCards, Human Gene Database. RND1 Gene: Rho Family GTPase 1. http://www.genecards.org/cgi-bin/carddisp.pl?gene=RND1

Kandasamy, SK; Larson, RG. Effect of salt on the interactions of antimicrobial peptides with zwitterionic lipid bilayers. *Biochimic et Biophysica Acta (BBA) Biomembranes* [Online] **2006**, 1758(9): 1274-84.

Li, L; Vorobyov, I; Allen, TW. The different interactions of lysine and arginine side chains with lipid membranes. *J Phy Chem B.* [Online] **2013**, 117(40): 11906-20.

Marcks myristoylated alanine rich protein kinase C substrate [Mus musculus (house mouse)]. *National Center for Biotechnology Information* [Online] **2016**.

Nobes, CD; Inger, L; Mattei, MG; Paris, S; Hall, A; Chardin, P. A New Member of the Rho Family, Rnd1, Promotes Disassemby of Actin Filament Structures and Loss of Cell Adhesion. *The Journal of Cell Biology*. [Online] **1998**. 141(1):187-97.

Oinuma, I; Kawada, K; Tsukagoshi, K; Negishi, M. Rnd1 and Rnd3 targeting to lipid raft is required for p190 RhoGAP activation. *Mol Biol Cell*. [Online] **2012**, 23(8): 1593-1604.

Okada, T; Sinha, S; Esposito, I; Schiavon, G; Lopez-Lago, MA; Su, W; Partilas, CA; Abele, C; Hernandez, JM; Ohara, M; Okada, M; Viagle, A; Heguy, A; Socci, ND; Sapino, A; Seshan, VE; Long, S; Inghirami, G; Rosen, N; Giancotti. The Rho-GTPase Rnd1 Suppresses Mammary Tumorigenesis and EMT by Restraining Ras-MAPK signaling. *Nat Cell Biol*. [Online] **2014**, 17(1): 81-94.

Ridley, AJ. Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking. *Trends in Cell Biology*. [Online] **2006**, 16(10): 522-9.

The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC.

Shi, X; Li, X; Kaliszewski, MJ; Zhuang, X; Smith, AW. Tuning the Mobility Coupling of Quaternized Polyvinylpyridine and Anionic Phospholipids in Supported Lipid Bilayers. *Langmuir* 31. **2015**, 1784-91.

Shi, X; Kohram, M; Zhuang, X; Smith, AW. Interactions and Translational Dynamics of Phosphatidylinositol Bisphosphate (PIP2) Lipids in Asymmetric Lipid Bilayers. *Langmuir* 32. **2016**, 1732-41.

Zhu, S; Liu, H; Wu, Y; Heng, BC; Chen, P; Liu, H; Ouyang, HW. Wnt and Rho GTPase signaling in osteoarthritis development and intervention: implication for diagnosis and therapy. *Arthritis Research & Therapy*. [Online] **2013**, 15: 217.

Safety Appendix

Safety training was taught by the graduate student Maryam Kohram, overseen by Dr. Adam Smith. Personal Protective Equipment (PPE) was followed to minimize the risk and occurrence of injuries and illnesses. Proper safety glasses and lab gloves were worn at all times. Proper training was required before use of all the equipment, including but not limited to: rotary evaporator, sonicator, NanoDrop, and spectrofluorimeter. Before handling the sulfuric acid and hydrogen peroxide, the Material Safety Data Sheets (MSDS) were overlooked. These compounds were used under a hood and a lab coat, extra gloves, and a face mask were always worn. The piranha mix was properly disposed of by waiting until unreactive and colder than room temperature and then added to the designated waste bottle. While no injuries or spills occurred, they would have been reported to Maryam, Dr. Smith, or another graduate student depending on the extent of the situation.